

## SUMMARY OF RESEARCH

**Title:**

INFLUENCE OF UNWEIGHTING ON INSULIN SIGNAL TRANSDUCTION IN MUSCLE

**Grant number:**

NAG2-1187

**Principal Investigator:**

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## ORIGINAL ABSTRACT

Unweighting of the juvenile soleus muscle is characterized by an increased binding capacity for insulin relative to muscle mass due to sparing of the receptors during atrophy. Although carbohydrate metabolism and protein degradation in the unweighted muscle develop increased sensitivity to insulin *in vivo*, protein synthesis *in vivo* and system A amino acid transport *in vitro* do not appear to develop such an enhanced response. The long-term goal is to identify the precise nature of this apparent resistance in the insulin signal transduction pathway and to consider how reduced weight-bearing may elicit this effect, by evaluating specific components of the insulin signalling pathway. Because the insulin-signalling pathway has components in common with the signal transduction pathway for insulin-like growth factor (IGF-1) and potentially other growth factors, the study could have important implications in the role of weight-bearing function on muscle growth and development. Since the insulin signalling pathway diverges following activation of insulin receptor tyrosine kinase, the immediate specific aims will be to study the receptor tyrosine kinase (IRTK) and those branches, which lead to phosphorylation of insulin receptor substrate-1 (IRS-1) and of Shc protein. To achieve these broader objectives, we will test *in situ*, by intramuscular injection, the responses of glucose transport, system A amino acid transport and protein synthesis to insulin analogues for which the receptor has either a weaker or much stronger binding affinity compared to insulin. Studies will include: a) estimation of the ED<sub>50</sub> for each analogue for these three processes, b) the effect of duration (1 to 4 days) of unweighting on the response of each process to all analogues tested, c) the effect of unweighting and the analogues on IRTK activity and d) the comparative effects of unweighting and analogue binding on the tyrosine phosphorylation of IRTK, IRS-1, and Shc protein.

## ACCOMPLISHMENTS

### Insulin analogues.

In July 1998 we received from Novo Nordisk 5 mg each of human insulin (HI) and 3 analogues designated as X2 ( $\beta$ -Asp<sup>9</sup>,  $\beta$ -Glu<sup>27</sup>), X10 ( $\beta$ -Asp<sup>10</sup>) and H2 ( $\alpha$ -His<sup>8</sup>,  $\beta$ -His<sup>4</sup>,  $\beta$ -Glu<sup>10</sup>,  $\beta$ -His<sup>27</sup>) based on the amino acid substitutions relative to human insulin. These analogs were selected because X2 reportedly binds with less affinity (K<sub>d</sub> 139% of HI) and X10 (K<sub>d</sub> 14% of HI) and H2 (K<sub>d</sub> 1.5% of HI) with significantly stronger affinities (Hansen et al. Biochem J 315:271-279, 1996). Their reported biological potencies corresponded qualitatively with their strength of binding though X10 and H2 had relatively much greater effects on growth (mitogenic) than metabolic processes.

### Measurement of ED<sub>50</sub>s

**Glucose transport:** We studied the effects of HI and the analogues on glucose transport, as measured by <sup>3</sup>H-2deoxyglucose uptake with <sup>14</sup>C-mannitol as the extracellular marker. The *in situ* glucose transport method previously had been used extensively by us, and this series of experiments proved to generate the best results. To inject the soleus, a 5 mm incision was made in the outer side of the shaved, ethanol-swabbed hindlimb of rats anesthetized with xylazine (8 mg/kg) plus ketamine (63 mg/kg). After the underlying fascia was cut, the soleus was exposed

by gently hooking it with fine forceps. The belly of the muscle was injected, as described below, using a 10  $\mu$ L Hamilton syringe equipped with a 26 gauge bevel-tipped needle. Incisions were covered by gauze wetted with warm (37° C) isotonic saline solution. Rats were then placed under an incandescent light bulb to help maintain their body temperature during the incubation period.

Uptake of 2-deoxyglucose was estimated in situ. Contralateral muscles were injected (4  $\mu$ L/100 g body wt) with a solution containing 20 mM 2-deoxy[1,2-<sup>3</sup>H]glucose, 0.1% bovine serum albumin (BSA), 150 mM NaCl, and <sup>14</sup>C-mannitol in the absence or presence of human insulin or one of its analogs at the indicated concentrations. These analogs included X2 (B-Asp<sup>9</sup>,B-Glu<sup>27</sup>) and H2 (A-His<sup>8</sup>,B-His<sup>4</sup>,B-Glu<sup>10</sup>, B-His<sup>27</sup>). In each rat, the left soleus served as the control (without insulin) while the right muscle received hormone treatment. Contralateral muscles were injected with labeled deoxyglucose in the absence or presence of one of the 4 hormones. Injected concentrations ranged from 10<sup>-11</sup> to 10<sup>-5</sup> M. In accord with previous in vitro studies the highest concentration (10<sup>-5</sup> M) tended to be inhibitory so was dropped from further studies. Human insulin was tested first to establish the procedure and for training the new personnel on the project. Unlike in vitro systems where the concentration in the medium is very consistent, the in situ technique lends itself to more variation because the injected hormone is diluted by the muscle interstitial space. Because of such variation more experiments, than expected, were required to estimate the ED50s for the effects of the hormones on glucose uptake.

*Protein synthesis:* This approach was finally established showing that it is a useful method for evaluating in situ muscle protein synthesis. Protein synthesis was estimated from the incorporation of [<sup>3</sup>H]phenylalanine into total protein in situ using the IM flooding dose approach. Muscles were injected (4  $\mu$ L/100 g body wt) with a solution containing 84 mM L-[2,6-<sup>3</sup>H]phenylalanine, 0.1% BSA, 110 mM NaCl in the absence or presence of human insulin or one of its analogs at the indicated concentrations. These analogs included X2 and H2 (Novo-Nordisk, Denmark). In each rat, the left soleus served as the control (without insulin) while the right muscle received hormone treatment. Twenty minutes after IM injection animals were killed by cervical dislocation. Then the middle approximately two-thirds of the soleus was excised. Previous experiments determined that 89  $\pm$  3% of injected radioactivity resided in this portion of the muscle. Consequently excision of the entire muscle would underestimate the process being measured. As for glucose transport we evaluated human insulin and its analogues for potency in promoting phenylalanine incorporation into protein.

*Results.* Analogs of human insulin have been used to discriminate between responses of metabolic and mitogenic (growth-related) pathways. This study compared the stimulatory effects of human insulin (HI) and two analogs (X2, B-Asp<sup>9</sup>,B-Glu<sup>27</sup> and H2, A-His<sup>8</sup>,B-His<sup>4</sup>,B-Glu<sup>10</sup>, B-His<sup>27</sup>) on glucose uptake and protein synthesis in rat soleus muscle in situ. Glucose uptake, estimated by intramuscular (IM) injection of 2-deoxy[1,2-<sup>3</sup>H]glucose with or without insulin, was maximally increased at 10<sup>-6</sup> M for HI and X2 and 10<sup>-7</sup> M for H2. HI had a larger effect (318%) than either X2 (156%) or H2 (124%). The ED<sub>50</sub> values for HI, X2 and H2 were 3.3x10<sup>-8</sup> M, 1.7x10<sup>-7</sup> M, and 1.6x10<sup>-9</sup> M, respectively. Protein synthesis, estimated by protein incorporation of [<sup>3</sup>H]phenylalanine injected into muscles with or without insulin, was maximally increased at 10<sup>-5</sup> M for HI and 10<sup>-6</sup> for X2 and H2. HI had a larger effect in stimulating protein synthesis (34%) than either X2 (25%) or H2 (19.8%). The ED<sub>50</sub> for HI, X2 and H2 were 3.0x10<sup>-7</sup>

M,  $3.2 \times 10^{-7}$  M, and  $1.0 \times 10^{-9}$  M, respectively. The biological potency of each analog ( $ED_{50} \text{insulin} / ED_{50} \text{analog}$ ) showed X2 to be less potent than HI for both glucose uptake (0.2) and protein synthesis (0.9) whereas H2 is more potent than HI with ratios of 20 and 300, respectively. These data suggest that this approach for studying insulin responsiveness in a single muscle in situ may be a useful tool for investigating insulin signaling in muscle in vivo.

*The above data are in press in:*

Randi B. Weinstein, Noura Eleid, Catherine LeCesne, Bianca Durando, Julie T. Crawford, Michelle Heffner, Christle Layton, Matthew O'Keefe, Jennifer Robinson, Suzy Rudinsky, Erik J. Henriksen and Marc E. Tischler. *Differential  $ED_{50}$ s of Human Insulin and its Analogs for In Situ Glucose Transport and Protein Synthesis in Rat Soleus Muscle*. Metabolism 51, in press (August 2002)

Comparative table from paper in press

Relative  $ED_{50}$  Values for Stimulation of Glucose Transport and Growth-related Pathways Compared for In Situ Studies in Skeletal Muscle and In Vitro Studies Using Primary Adipocytes or CHO Cells.

Analog	Relative ED <sub>50</sub> (HI ED <sub>50</sub> /analog ED <sub>50</sub> )				Relative K <sub>d</sub> (%)
	Glucose transport		Growth-related pathways		
	In situ	In vitro	In situ	In vitro	
X2	0.2	0.4	0.9	0.6	139
H2	20.6	4.3	300.0	29.5	1.5

NOTE. In situ muscle data are taken from Figs 1 and 2. In vitro data using primary adipocytes (glucose transport, uptake of 3-O-methylglucose) and CHO cells (growth-related, thymidine incorporation into DNA or Relative  $K_d$ , analog  $K_d$ /HI  $K_d$ ) are shown for these same analogs.

### Unweighting Studies:

The effects of human insulin and analogues X2 and H2 were compared on glucose transport and protein synthesis, as measured above, in soleus muscles unweighted for 1, 3 or 4 days using the  $ED_{50}$  concentration determined above. Values were obtained at all time points. The data are still under analysis and if the results are satisfactory will be prepared for publication. Preliminary conclusions suggest that while unweighted soleus showed an enhanced effect of insulin on glucose transport, there may not be a similar effect on protein synthesis. Preliminary analysis also suggests that this differential is particularly true for the analogue that binds more weakly. However until further analysis the results are not conclusive.

### **Studies of Insulin Signaling Components:**

Unfortunately this area of work was not sufficiently successful to reach any conclusions though the studies did allow us to improve the techniques needed to assess the effects of analogues on the glucose transporter, and insulin receptor tyrosine kinase. This work is continuing in collaboration with Dr. Erik Henriksen as part of Mr. Matt O'Keefe's graduate dissertation project. Materials to continue this work were obtained before the termination of this grant and any work that results from that will be submitted as a report addendum at a future date. Any publications that derive from those studies will cite this NASA grant as providing partial support. Dr. Tischler will continue as a collaborator on the project and Dr. Henriksen's support will provide the animals and additional materials to continue these experiments even though this grant has terminated.

### **Personnel who participated on the Project:**

#### Faculty -

Erik Henriksen, PhD – associate professor of Physiology (collaborator)  
Randi Beth Weinstein, PhD - research assistant professor  
Michael Wells, Ph.D. – professor of Biochemistry (consultant)

#### Graduate students -

Ilene Brodsky  
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#### Undergraduate students -

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**NO SUBJECT INVENTIONS RESULTED FROM THE WORK OF THIS GRANT.**